(12) UK Patent Application (19) GB (11) 2 228 736 (13) A

(43) Date of A publication 05.09.1990

(21) Application No 9001733.6

(22) Date of filing 25.01.1990

(30) Priority data
(31) 308624 (32) 10.02.1989 (33) US

(51) INT CL[5]
C08L 5/08, A61K 7/48

(52) UK CL (Edition K)
C3U UFB U207 U522 U523
A5B BFH B161
U1S S1343

(56) Documents cited
GB 2099826 A EP 0338459 A EP 0283893 A
EP 0258558 A EP 0237644 A WO 88/04931 A
JP 59116207 A

(58) Field of search
UK CL (Edition J) C3U UFB UFZ
INT CL[4] C08L
Online: WPI

(71) Applicant
Sterivet Laboratories Limited

(Incorporated in Canada - Ontario)

3909 Nashua Drive, Mississauga, Ontario, L4V 1R3, Canada

(72) Inventor
Alan Drizen

(74) Agent and/or Address for Service
Venner Shipley and Co
368 City Road, London, EC1V 2QA, United Kingdom

(54) Cosmetic formulation

(57) A heat stable, purified, heat-sterilized fraction of hyaluronic acid is disclosed which is suitable for use in cosmetic formulations for application to the skin. A formulation for such cosmetic use includes an aqueous solution of the heat stable fraction of hyaluronic acid and preservatives.

GB 2 228 736 A

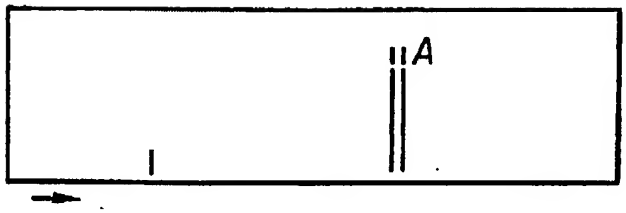


FIG. 1

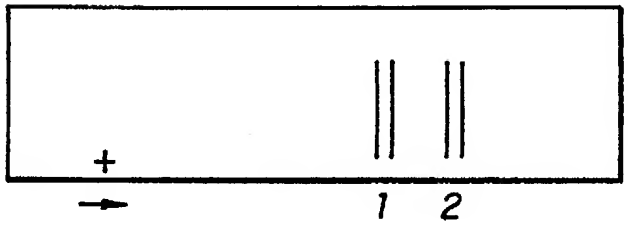


FIG. 2

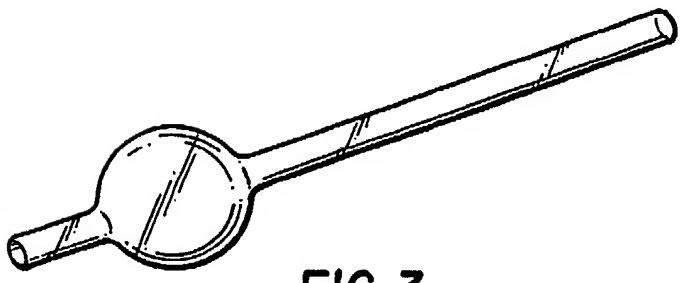
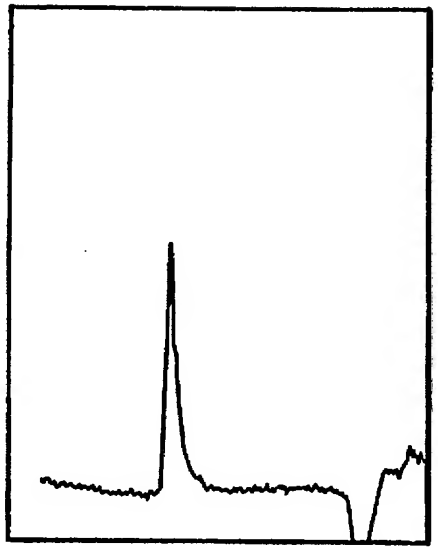
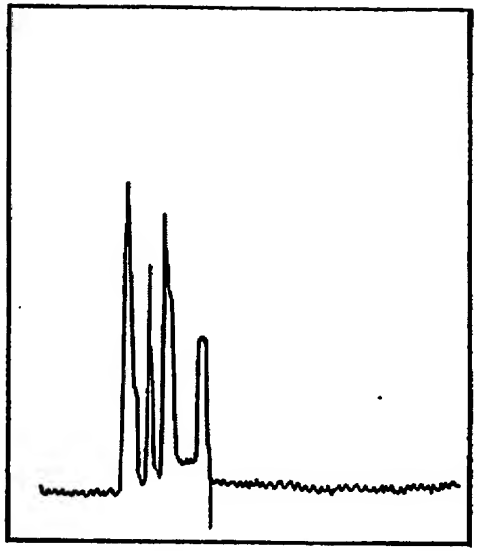


FIG. 3



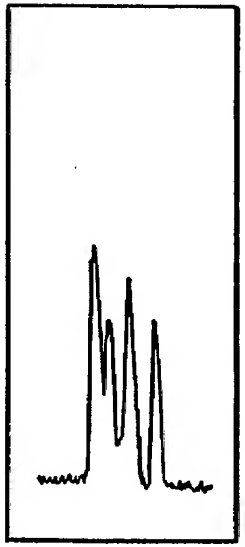
VISIBLE YOUTH
BIO POLYMERS

FIG. 4A



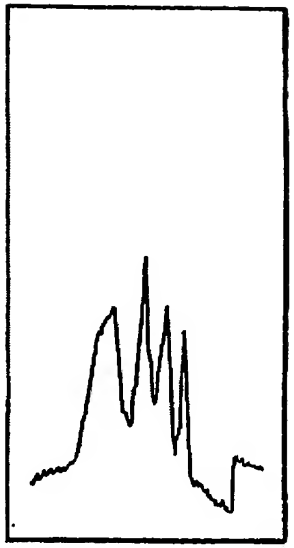
B.H-24 DAY ESSENCE
SHISEIDO

FIG. 4B



B.H-24 NIGHT
ESSENCE
SHISEIDO

FIG. 4C



NIGHT REPAIR
ESTEE' LAUDER

FIG. 4D

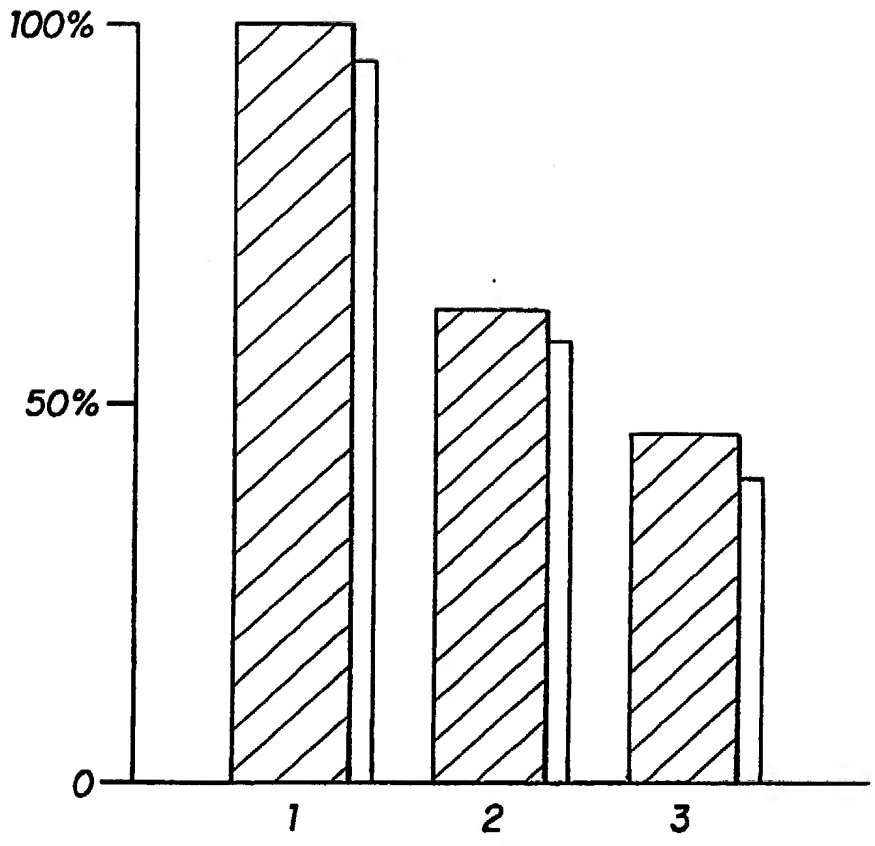


FIG. 5

COSMETIC FORMULATIONS AND ISOLATION AND PURIFICATION OF FRACTIONS OF SODIUM HYALURONATE USED IN COSMETIC FORMULATIONS

DESCRIPTION

The present invention relates to cosmetically useful preparations based on hyaluronic acid, a naturally-occurring substance, which is found in animal tissue, and especially in rooster comb, in addition to vitreous humor, umbilical cords, and synovial fluid of mammals. More particularly, the present invention is directed to purified sodium hyaluronate; processes of isolating and purifying sodium hyaluronate; formulations and compositions containing sodium hyaluronate suitable for cosmetic use; and methods of preparing a formulation containing such fraction of hyaluronic acid suitable for this use, in addition to and methods for making and using such formulations and compositions containing such fraction of hyaluronic acid for cosmetic purposes.

The natural function of healthy skin is to provide protection against a wide variety of potentially harmful environmental influences. Healthy skin, therefore, is both physically strong and at the same time capable of blocking out injurious wavelengths of sunlight and also harmful bacteria. In addition, healthy skin is also capable of acting as a protective barrier against constant assault by a wide variety of dangerous airborne chemical pollutants ever present in most modern cities.

The mechanical strength of healthy skin is due at least in part to a network of highly specialized protein

fibers which crisscross the entire structure of skin in all directions. Collagen and elastin are two of the substances which are part of the composition of these fibers. This network of fibers, called connective tissue, helps to protect the skin's blood vessels, nerves and living cells against assault by environmental factors, both large and small, related to everyday life.

Gaps between the meshes of this connective tissue are filled with a jelly-like mixture of mucopolysaccaride (sugar related) substance known as hyaluronic acid (hyaluronate) and water. The presence of the hyaluronate/water complex within the connective tissue is essential for the transport of nourishment to the living cells of the skin. The ability of the skin to maintain an effective barrier against bacteria and pollutants from the outside world depends on the health of the hyaluronate/water complex. Equally as important is the hyaluronate/water complex's ability to prevent dryness, rough skin, lines and aging.

The hyaluronate/water complex makes up, by far, the largest part of total skin volume and any temporary or lasting reduction in the quality of hyaluronate in the hyaluronate/water complex will result in dry skin, lines and the appearance of aging of the skin.

Hyaluronic acid (HA) is also known as a glycosaminoglycan. The repeating unit of the hyaluronic acid molecule is a disaccharide consisting of D-glucuronic acid and N-acetyl-D-glucosamine. Because hyaluronic acid possesses a negative charge at neutral pH, it is soluble in water, where it forms highly viscous solutions. The D-glucuronic acid unit and N-acetyl-D-glucosamine unit are bonded through a glycosidic, beta(1---3) linkage, while each disaccharide unit is bonded to the next disaccharide unit through a beta(1---4) linkage. The beta(1---4) linkages may be broken through hydrolysis with the enzyme hyaluronidase.

A variety of substances, commonly referred to hyaluronic acid, have been isolated by numerous methods from various tissue sources including umbilical cords, skin, vitreous humour, synovial fluid, tumors, haemolytic streptococci pigskin, rooster combs, and the walls of veins and arteries.

Conventional methods for obtaining hyaluronic acid result with a product having differing properties and a wide range of viscosities. U.S. Patent 2,585,546, HADIAN, is an example of a method for obtaining hyaluronic acid which involves extracting acetone-washed umbilical cords with a dilute salt solution, acidifying the resulting extract, removing the clot so formed, precipitating some hyaluronic acid with protein from the acidified extract with ammonium sulfate, agitating the liquid with pyridine, precipitating another fraction highly contaminated with protein, followed by more ammonium sulfate which forces some pyridine out of solution along with the high viscosity hyaluronic acid. The hyaluronic acid collects at the interface between the two liquid phases and may be separated by filtration, centrifugation or other usual procedure. A modification of this process involves the fractionation of the acidic salt extract from umbilical cords with alcohol and ammonium sulfate. Alcohol is added to the acidic salt extract, and the resulting precipitate is removed. Solid ammonium sulfate is added to the liquid until saturation and the solution forms two phases with a precipitate of hyaluronic acid at the interface.

U.S. Patent 3,396,081, BILLEK, produces a dry powder of hyaluronic acid obtained from a source of hyaluronic acid, such as animal organs including the vitreous body of the eye, umbilical cords, and the like, or from bacterial cultures producing hyaluronic acid. A suspension of the resultant dry powder is heated in water for a short time in an alkaline range whereby the protein content is denatured. After adjustment of the optimum pH and temperature range

for the enzyme used, the protein is decomposed by proteolytic ferments, preferably by a hydrolase mixture of Aspergillus oryzae. After removing the free amino acids and mineral salts by treatment with ion exchanges, an impure hyaluronic acid solution still containing protein is obtained. The resultant solution of impure hyaluronic acid containing residual protein is then adjusted to an acid pH of about 3-4 in which the impurities form with hyaluronic acid an insoluble complex, while part of the hyaluronic acid itself functions as precipitate for the impurities, particularly for the residual proteins which can otherwise be removed only with difficulty without depolymerization of the hyaluronic acid, whereupon the resulting insoluble complex compounds are separated by high speed centrifuging. After a sodium salt, the resultant hyaluronic acid solution with a concentration of 0.2% in water has a relative specific viscosity of 20 and constitutes a water-clear solution disclosure as being free from proteins, antigens, and pyrogens, which is disclosed as being suitable for heat sterilization without experiencing a considerable drop in viscosity.

U.S. Patent 3,862,003, OKUYAMA et al., is directed to a method of extracting mucopolysaccharides from connective tissues of animals, which involves exposing the connective tissue with water at a temperature of 105°-150°C under an elevated pressure, subjecting the resultant extract to a protease treatment and/or alkali treatment, and then separating and recovering mucopolysaccharides.

U.S. Patent 4,141,973, BALAZS, is directed to the production of an ultra-pure, high molecular weight hyaluronic acid fraction which is obtained from animal tissue containing hyaluronic acid by a process which involves moving blood from the animal tissue containing hyaluronic acid, extracting hyaluronic acid from the blood, deproteinizing the hyaluronic acid extract, and removing any unidentified inflammation causing agents present

therein by treating the deproteinized hyaluronic acid extract at a pH of 6.0-7.0 with a volume of chloroform at least about equal to that of the deproteinized extract, to form a two-phase mixture which is then stirred, sufficiently to insure intimate contact with the two phases at about 15°-40°C, followed by separating out and discarding the chloroform phase.

U.S. Patent 4,517,296, BRACKE et al., is directed to the preparation of hyaluronic acid in high yield from streptococcus bacteria by fermenting the bacteria under anaerobic conditions in a CO_2 enriched growth medium, separating the bacteria from the resulting broth and isolating the hyaluronic acid from the remaining constituents of the broth. Separation of the microorganisms from the hyaluronic acid is facilitated by killing the bacteria with trichloroacetic acid. After removal of the bacteria cells and concentration of the higher molecular weight fermentation products, the hyaluronic acid is isolated and purified by precipitation, resuspension and reprecipitation. Further material relevant to this application is disclosed in U.S. Patent Application No. 0541859, which is incorporated herein by reference.

The present invention overcomes the difficulties of the prior art through the discovery of a hyaluronic acid preparation which contains HA fractions which are heat stable, purified, and heat-sterilized. The HA fractions have been found to be effective for use in cosmetic preparations and formulations intended for use in skin care treatment.

According to the present invention there is provided a cosmetic formulation for topical administration to epidermal tissue which is composed of a fraction of hyaluronic acid, preferably wherein the hyaluronic acid is present in the form of a salt, such as sodium salt, e.g., sodium hyaluronate, and at least one preservative, preferably selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of

sodium benzoate, methylparaben, and propylparaben, but more preferably methyl paraben alone.

In a preferred embodiment the present invention provides a formulation, as described above, including, about 1-1.2% of a sodium salt of hyaluronic acid, and about 0.2-0.25% methyl paraben; and preferably about 1.0% of the sodium salt of hyaluronic acid, i.e., sodium hyaluronate, about 0.2% of methylparaben with the remainder, i.e., 98-99% being water.

In a second aspect, the present invention provides a fraction of hyaluronic acid, useful in the cosmetic formulations, as described above, wherein the fraction of hyaluronic acid has an average molecular weight of less than 800,000, preferably greater 500,000, more preferably wherein the average molecular weight is within the range of about 600,000 to 700,000, and most preferably within the range of about 625,000-675,000

In a further aspect, the present invention provides a fraction of hyaluronic acid, useful in cosmetic formulations as described above, which has one or more of the following characteristics:

a) contains less than 1.25 mg., and preferably less than 0.5 sulfated mucopolysaccharide per 100 mg hyaluronate as determined by an extinction reading not exceeding 0.018;

b) contains not more than 15 ppm, and preferably less than 100 ppm, iron as determined by atomic absorption spectometry;

c) contains not more than 15 ppm, and preferably less than 10 ppm, lead as determined by atomic absorption spectometry;

d) based on 300 mcg. sodium hyaluronate, contains the following:

i) glucosamine at less than 0.75 mcg., and preferably less than 0.5 mcg.;

ii) glucuronic acid at less than 7.5 mcg., and preferably less than 5.0 mcg.;

iii) N-acetylglucosamine at less than about 7.5 mcg. and preferably less than about 7.5 mcg. and preferably less than about 5.0 mcg; and

iv) amino acids at less than about 0.15 mcg. and preferably less than about 0.5 mcg;

e) a protein content of less than about 0.6%, and preferably less than about 0.4%, using the Lowry method;

f) a UV extinction coefficient at 257 nm of less than about 0.275, and preferably less than about 0.23.

g) a UV extinction coefficient at 280 nm of less than about 0.25, and preferably less than about 0.19.

h) a pH within the range of 7.3-7.9 and preferably within the range of 7.5-7.7.

In a yet further aspect, the present invention provides a method for preparing a cosmetic formulation for topical administration to epidermal tissue which involves providing such fraction of hyaluronic acid, preferably sodium hyaluronate, as described above; filling a mixing tank with hot water, e.g., heated to a temperature of about 60°C-90°C; introducing and dissolving preservatives selected from the group consisting of methyl parahydroxybenzoate, propyl parahydroxybenzoate, sodium benzoate and mixtures of methyl parahydroxybenzoate, propyl parahydroxybenzoate and sodium benzoate, but more preferably methyl paraben, in the water to form a preservative solution; adding and dissolving the fraction of hyaluronic acid in the preservative solution to form a resultant solution; adjusting the pH of the resultant solution to 7.5-7.7; diluting the pH adjusted solution with an amount of water to a final volume aqueous formulation; filling the aqueous formulation into vials; preferably which also involves filtering the final volume of aqueous formulation through a membrane into a receiving vessel prior to filling into the vials, preferably wherein said membrane is a 0.8 micron membrane.

Fig. 1 is an electrophoresis strip showing a resultant single band of blue color, representing hyaluronic acid, as detected by the electrophoretic identification of hyaluronic acid, discussed herein.

Fig. 2 is a similar electrophoretic strip, similar to the one shown in Fig. 1, which shows an additional characteristic blue band of sodium hyaluronate, i.e., which is a relatively weak band with a higher Rf.

Fig. 3 shows a glass reflexing apparatus used to determine the sulfate content of mucopolysaccharides which may be present in the hyaluronic acid.

Fig. 4 illustrates graphs comparing the analysis of current hyaluronate-based products using high pressure liquid chromatography (HPLC) methods.

Fig. 5 is a chart showing a comparison of purity levels of hyaluronic-based products.

The present invention is based on the discovery that poor skin quality caused by depleted or reduced levels of hyaluronate can be remedied by the application of the formulation for treatment of skin conditions containing a fraction of hyaluronic acid in accordance with the present invention to dry or damaged skin. The extraordinary absorption qualities of the formulation for treatment of skin conditions containing a fraction of hyaluronic acid in accordance with the present invention have been said to be apparent on the first application. Although not wishing to be bound by any theory, the formulation for the treatment of skin conditions in accordance with the present invention is believed to penetrate into the deep layers of the skin to replace the skin's natural hyaluronate, lost through environmental damage and age.

The formulation for treatment of skin conditions in accordance with the present invention has been observed to provide deep moisturizing and other anti-aging benefits to

dry, lined and otherwise damaged skin. Containing a novel and unique molecule of natural hyaluronate developed especially for skin use, the formulations for treatment of skin conditions in accordance with the present invention are substantially totally absorbed into the deepest layers of skin where, because the molecular structive of the fraction of hyaluronic acid used in accordance with the present invention is substantially identical to that contained in healthy skin, it replenishes the lowered concentration of hyaluronate of dry, damaged and aging skin.

The formulations for treatment of skin conditions in accordance with the present invention provides essential natural hyaluronate, to rehydrate aging, damaged or dry skin. Without the formulations for treatment of skin conditions in accordance with the present invention it is virtually impossible to restore the hyaluronate/water complex found in normal skin.

The present invention is, therefore, based on the discovery of a novel and unique molecule of hyaluronic acid developed especially for skin care and possesses characteristics not found in any other hyaluronate-based formulation. The hypo-allergenic formula for treatment of skin conditions in accordance with the present invention is believed to contain the purest form, substantially, 100% of hyaluronate available. Although not wishing to be bound by any theory, it is believed that hyaluronate, a natural occurring moisture-binding substance found in skin and connective tissue, actually repairs skin ravaged by the effects of sun, wind, and aging. A small application of the formula for treatment of skin conditions in accordance with the present invention has been found to improve skin quality and appearance, reduce wrinkles, and replenish skin.

In addition to being a superior skin care formula, the formula for treatment of skin conditions in accordance with

the present invention has been found to offer uncommon versatility and convenience. Proper concerns for its purity factor (100%) absorbability, efficacy and visco elasticity (ease of application) distinguishes the formula for treatment of skin conditions in accordance with the present invention as an ideal skin care product. Coupled with its non-allergenic properties, the formula for treatment of skin conditions in accordance with the present invention is not only an exceptional overnight skin treatment, but a perfect base for all types of make-up.

The anti-aging benefits of the formulations for treatment of skin conditions in accordance with the present invention include: rejuvenation of damaged skin; provision of deep moisturizing, total absorption; allergy free, colour, fragrance and compatibility with make-up and skin cremes, in addition to containing substantially 100% pure natural hyaluronate.

Pure, natural hyaluronate is a relatively new substance for skin care. As previously mentioned, hyaluronate have special characteristics of visco elasticity, lubrication and cell nourishment which properties make the formulations for treatment of skin conditions in accordance with the present invention and its active ingredient, hyaluronate, invaluable anti-aging agent.

Although not wishing to be bound by any particular theory, the formulations for treatment of skin conditions in accordance with the present invention contains natural hyaluronate which attaches itself to human skin and is extremely hydrophilic, thereby hydrating the skin. Also, air penetrates the formulations for treatment of skin conditions in accordance with the present invention, thus allowing the skin to breath while being moisturized. Like elastin and collagen the active, ingredient of the formulations for treatment of skin conditions in accordance with the present invention, i.e., hyaluronate, is a

naturally occurring component of human skin. It influences skin structure by forming bonds between collagen fibrils and retaining moisture like a molecular sponge. The total effect of the formulations for treatment of skin conditions in accordance with the present invention is to increase blood circulation and accelerate the supply of nutrients to the skin. In this way, the formulations for treatment of skin conditions containing hyaluronate in accordance with the present invention improves colour, tone and texture of the skin resulting in a healthier look and softer feel.

Prior to the present invention, it is not believed that the molecule of hyaluronate designed specifically for skin care as incorporated in the formulations for treatment of skin conditions in accordance with the present invention has been produced.

The following is a detailed description of the preferred procedures for producing hyaluronate and the formulations for treatment of skin conditions containing hyaluronate in accordance with the present invention.

Although isolation and purification of sodium hyaluronate from rooster combs is the best mode for purposes of the present invention, it should be understood, that other sources of hyaluronic acid may be used for purposes of the present invention.

Prior to being selected roosters are inspected and declared healthy before rooster combs, weighing approximately 20 - 80 g, are cut from the head of recently decapitated birds and processed in an automated processing plant. Within 30 minutes the combs are quick frozen at preferably -40°C using a cold-plate technique. Following freezing the combs are placed in plastic bags and packed in cardboard boxes. The frozen "blocks" of rooster combs, which weigh between 2 and 5 kg, are stored at -20 to -40°C during transport and are thereafter maintained at -20 to -40°C in freezers equipped with continuous recorders, alarm and automatic restart systems during storage. Although the

combs may be stored frozen without loss of hyaluronic acid content for up to 12 months before further processing, there is a storage limit of 8 months from date of receipt.

Preferably, 3000 kg of frozen rooster combs are processed at one time and are assigned a separate batch number. A sufficient quantity of combs are transported to an air-conditioned process room with a filtered air supply which is maintained under strict sanitary conditions. After partial thawing in suitable containers the combs are thoroughly washed with tap water. After washing, the combs are weighed and minced in a stainless steel (S-S) 316 meat grinder, with 4 mm diameter holes.

The minced combs are pumped through a S/S lobed pump and S/S feeder lines to a 10,000 l S/S vessel equipped with a speed regulated stirrer, and mixed with anhydrous acetone, pumped from an external storage tank through a flow meter. All parts in contact with acetone are made of 316 S/S. Acetone, in the proportion of 2:1 (v/v) of acetone to minced combs, is added to the vessel and stirred at 50 rpm for 6 hours. The proportion of acetone to fresh tissue is particularly important, since the first 3 dehydration steps removes water soluble impurities. The acetone-minced comb mixture is left to settle for approximately 12 hours after which time the supernatant acetone is siphoned off and discarded (acetone is retained and is recovered for re-use by distillation).

The acetone extraction is repeated at least 6 times until a clear, colorless acetone supernatant, containing less than 5% moisture, is obtained. In so doing, the water content of the acetone is analyzed as an in-process control procedure using a Karl-Fischer method and the results are recorded on the manufacturing work sheet. If the 6th acetone extract does not meet the 5% moisture specification, the acetone extraction is repeated.

After the final acetone extraction, the wet, minced cake is pumped, while maintaining stirring, through an Alfa

Comi Condor, continuous flow centrifuge to produce a dried product having a moisture content within the range of about 1-3%, preferably about 1-2% and more preferably about 1%. The dried product is collected in suitable containers, labeled as to batch, total weight and date of drying. At this stage the material may be stored for 90 days at 17 - 18°C before further processing. The above procedure yields 500 - 600 kg of dried powder from the starting 3000 kg of fresh rooster combs.

250 - 300 Kg of dried rooster comb powder, produced above, is mixed in a jacketed S/S vessel with 3000 l of a cysteine-phosphate buffer, pH 7.5, prepared as follows:

$Na_2HPO_4 \cdot 12H_2O$	30.750 g	
$NaH_2PO_4 \cdot 2H_2O$	5.850 g	
Cysteine HCI		5.400 g
Methyl/ethyl parabens		2.500 g
Non-pyrogenic, deionized water prepared by reverse osmosis using a Millipore membrane RO-60		to 3000 l

Using an automatic thermal regulator, the phosphate buffer-tissue mixture is brought to 60 - 65°C, while maintaining constant stirring at 60 rpm, and is maintained at that temperature for 1 hour. To the agitating mass is added a preparation of papain prepared by adding 200 g of crystalline papain with a minimum specific activity of 60,000 proteolytic units/g in a 5 liter glass carboy containing 4 liter of deionized water.

The enzyme - buffer - tissue mixture is agitated at 60 rpm for 24 hours at 60 - 65°C. The mixture is then cooled by circulation of cold water through the vessel jacket to 25°C, while maintaining stirring. To the mixture is added 60 kg of Celite-535 as a filtering aid and stirring is continued for 1 hour, after which time the mixture is filtered under pressure through a filter press. A clear yellowish liquid is obtained which is collected in a 5000 l

S/S vessel. The protein content should range between 30 - 40 mg/ml when measured using the Biuret reaction.

The clear, yellowish liquid is pumped through a S/S membrane ultrafiltration unit (4DDS, Denmark) with a total filter surface area of 42.6 m^2 and a membrane with a molecular exclusion limit of 30,000 daltons. The first ultrafiltration step is run at constant volume by the continuous addition of deionized water. A colorless ultrafiltrate is obtained equal to 5 to 6 times the original volume. Preferably after the 5th volume of ultrafiltrate is obtained, the absence of amino acids is checked using a ninhydrin assay. Ultrafiltration is continued until the permeate is negative for amino acids. After completion of the ultrafiltration the volume is reduced to 1/3 the original volume by continuing ultrafiltration without addition of water to produce a concentrated liquid.

The ultrafiltration step is important because small MW molecules such as amino acids, peptides, and nucleotides, are removed which may have inflammatory properties, thereby providing a product free of inflammatory activity.

After ultrafiltration the solution must be clear, its volume accurately recorded and the glycosaminoglycan (GAG) titre measured using a carbazole assay. The GAG level should be 12 - 17 mg/ml.

The concentrated liquid is then transferred to a 5000 1 S/S vessel equipped with a speed regulated agitator and constant temperature control. Sufficient NaCl (crystalline) is added with stirring to bring the solution to 0.1 Molar. The molarity is checked using a silver nitrate assay for chloride content. The mixture is heated to 50°C at the rate of 100 1//minute with agitation at a constant speed of 60 rpm.

Cetylpyridinium chloride solution (CCP) is prepared in a separate vessel by the addition of 45.0 kg cetylpyridinium chloride to 3000 1 of 0.1M sodium chloride

in deionized water. The solution is added with stirring to the aqueous tissue extract. Agitation is continued at 50 rpm for 60 minutes during which time a precipitate forms. 50 Kg of Celite-535 is added and mixed into the vessel. The mixture is cooled to 25°C over a period of time up to about 4 hours after which it is centrifuged at a constant speed of 250 rpm in 850 mm S/S baskets. The clear, liquid phase is discarded and the precipitate is transferred to a suitable S/S container.

The molarity control and CCP precipitation steps are important for the removal of glycoprotein.

The resultant precipitate is dissolved in a 5000 1 S/S vessel with an aqueous solution of 0.01M sodium chloride which has been prepared in a separate container containing 0.05% CCP. The mixture is agitated for 60 minutes at 100 rpm while being maintained at 50°C. The mixture is then cooled to 25°C over 4 hours and centrifuged as described in step 40. This washing procedure is repeated preferably at least two or three times. The final, washed precipitate is centrifuged at 800 rpm for 60 minutes (to separate the residual glycoprotein contaminants) and the precipitate transferred to a 5000 1 S/S vessel.

To the washed precipitate is added 3000 1 of 0.05M sodium chloride solution containing 0.05% CCP while maintaining agitation at 50 rpm and a temperature of 50°C for 60 minutes. The mixture is cooled to 25°C over 2 hours. The mixture is centrifuged as before and the clear supernatant liquid discarded. This procedure is repeated using a sodium chloride concentration of 0.1 Molar and 0.05% CCP. The mixture is centrifuged as before and the clear supernatant discarded. This procedure using 0.1M NaCl with 0.05% CCP is repeated at least once more and the washings discarded. The extraction of the precipitate from this step is carried out at least about three or four times using 0.25M sodium chloride, with each step using smaller volumes of 3000 1, 500 1, 500 1 and 500 1, respectively,

with the hyaluronic acid being measured in each extract. The hyaluronic acid content ranges of the extracts are 2 - 3, 1 - 3, 0.2 - 1 and 0 - 0.6 mg//ml, respectively, with the hyaluronic acid being measured in each extract. The hyaluronic acid content ranges of the extracts are 2 - 3, 1 - 3, 0.2 - 1 and 0 - 0.6 mg/ml, respectively. The extract supernatants are transferred into a 5000 l S/S container and the precipitate discarded.

This procedure is important to the isolation of pure hyaluronic acid and the elimination of other CCP complexing mucopolysaccharides.

The extract supernatant is raised in temperature to about 50°C with agitation and NaCl is added to bring the mixture to about 0.33 Molar. 1.0 Kg of CCP is added while maintaining agitation and a temperature of about 50°C for about 12 hours. The mixture is cooled to about 25°C for a period of time up to about 5 hours during which time the precipitate is allowed to settle. The precipitate which forms contains of impurities and is collected by filtration through a filter press on Celite-535 coated pads. The clear, aqueous solution is re-filtered under pressure through an 0.7 micron filter (Pall, Gr. Britain) and the supernatant is separated for subsequent ultrafiltration.

The supernatant liquid is ultrafiltered at constant volume while maintaining the solution at 0.33M in sodium chloride until the equivalent of 3 initial volumes have been filtered. The filtrate is then concentrated by further ultrafiltration using a membrane with exclusion limits of 300,000 DDS.

This step eliminates low molecular weight fractions of hyaluronic acid which may be linked to peptides.

The concentrated solution is precipitated under agitation at about 60 rpm for about 4 hours at a controlled temperature of about 20°C with 2 volumes of 95% ethanol. The precipitate is allowed to settle and is collected by centrifugation and the supernatant discarded. The

precipitate is dissolved in 1000 l of 0.1M sodium chloride solution and is re-precipitated with 3 volumes of 95% ethanol using the same conditions as in the previous step. The precipitate is then collected and washed three times with 75% ethanol using 500 l, 250 l and 250 l volumes, respectively, and then with absolute ethanol three times with volumes of 500 l, 250 l and 250 l. The precipitate is then washed three times with 500 l, 250 l and 250 l volumes of anhydrous acetone.

This phase is important in the purification of the hyaluronic acid from the CCP complex and from other substances which can be carried through the previous steps.

The solid precipitate material is then dried under vacuum at 1 - 10^{-2} Torr and 30°C for 60 hours. This procedure yields 2.0 - 2.5 kg per 1000 kg fresh rooster combs of sodium hyaluronate. The specifications of reagents used in the purification of hyaluronic acid are listed below:

Acetone:	U.S.P.XX
Sodium Chloride:	U.S.P.XX
Cetylpyridinium Chloride:	U.S.P.XX
Ethanol:	U.S.P.XX - Denatured by the addition of 1% Toluol
Water:	Tap water is purified by reverse osmosis to an electrical conductivity value of 30 micro ms.

It is believed that the absence of histamine-like substances in the hyaluronic acid produced in accordance with the previously described method renders the purified sodium hyaluronate particularly suitable for cosmetic formulations. Related to this the absence of histamine-like substances in hyaluronic aced may be determined using the following procedure:

The assay of histamine-like substances assay has been carried out on several successive lots of material. Data for three lots follows, indicating the absence of such substances. The test used is the guinea-pig intestinal strip preparation described in most pharmacopoeias. One guinea pig, weighing between 200 and 350 g, is sacrificed (cranial trauma) and bled. The ileum is excised from the point of ileo-coecal conjunction. The first 10 cm of small intestine are discarded because of the presence of Peyer plaques which would render the preparation extremely unstable due to the histamine contained in the plaques. The piece of intestine is thoroughly washed with a pre-oxygenated solution kept at a higher temperature than room temperature. This solution, which is also used for the incubation of the strip, is composed as follows:

Concentrated physiological saline (50ml)
formulated as follows:

NaCl	8 g
KCl	0.2 g
$CaCl_2$ $2H_2O$	0.1325 g
$MgCl_2$ $6H_2O$	0.1065 g
Na_2HPO_4 $2H_2O$	6.25 g
$NaHCO_3$	1 g/l
glucose	0.5 g/l
atropin sulfate	0.5 mg/l
morphine hydrochloride*	5 mg/l
H_2O redistilled	950 ml

(*) morphine is added, in addition to Eur. Ph. specifications, in order to improve the stability of the organ preparation, suppressing spontaneous movement. Thus a very stable baseline is achieved without bias between peristaltic movements and response movements to exogenous histamine).

A 4-cm strip of ileum is fastened at both ends with a thin silk thread, without occluding the intestinal lumen.

One end is tied to the bottom of the incubation bath in which the organ is oxygenated by a flow of a mixture of O_2 and CO_2 (95% + 5%). The temperature of bath is kept constant at 37°C. The other end is tied to an isotonic transducer connected to an instrument for the recording of oscillations. After oxygenation, the incubation liquid has a pH of 7.3 - 7.4. After 1 hour rest, during which the preparation is repeatedly washed, the test can start.

Organ contraction is induced by repeated addition of histamine dihydrochloride (final concentration 0.1 mcg//ml) every 10 minutes until 2-3 equal responses are obtained. Repeated washings take place after a maximum contraction has been induced, which is monitored on the kymograph recorder, so as to allow return to base-line levels (maximal distention). Every 5 minutes, additional thorough washings take place. The histamine used is dissolved in the incubation liquid and before addition of the amount which will give the final concentration of 0.1 mcg/ml (0.2 ml) the same quantity of liquid is withdrawn from the bath.

When base-line is established, a test sample of Na-hyaluronate in a 0.5 ml volume (after withdrawal of 0.5 ml from the bath) is added. The test sample is made up from a 10 mg/ml solution in 0.9% NaCl. Since the incubation bath has a capacity of 6.5 ml, the final concentration of Na-hyaluronate reached in the bath is 769.23 mcg/ml. During 10 minutes the absence of contractions is recorded after which, without washing, 0.1 mcg/ml histamine is introduced into the bath. The resulting contraction should be equal to the contractions induced previously in the absence of Na-hyaluronate.

In the absence of a histamine-like response of the preparation after addition of the test sample, the lot represented is declared free of histamine-like substances.

Sodium hyaluronate produced in accordance with the present invention and used in cosmetic formulations is a soft, white, odorless, hygroscopic powder, which is water

soluble, non-dialyzable and insoluble in organic solvents, and may be identified by electrophoresis on cellulose acetate strips in which migration of the sample is compared to that of a standard with visualization using alcian blue. The presence of sodium is detected in the sulfated ash of hyaluronic acid using atomic absorption spectrophotometry. The presence of sulfated mucopolysaccharides, such as chrondroitin sulfate, may also be detected using the previously mentioned electrophoretic method. The sulfated compounds have a higher Rf than hyaluronic acid. The presence of sulfated mucopolysaccharides is quantitated by measuring the sulfate content of a perchloric acid digest of sodium hyaluronate using the analytical method described herein below.

Preferably, the sodium hyaluronate contains no more than 0.5 mg sulfated mucopolysaccharide per 100 mg sodium hyaluronate determined as an extinction reading not exceeding 0.018 (100 mcg SO_4 has an extinction of 0.18 at 660 nm), no more than 100 ppm of iron as determined by atomic absorption spectrometry, and no more than 10 ppm of lead as determined by atomic absorption spectrometry. Using thin-layer chromatography the presence of free glucuronic acid, glucosamine and N-acetylglucosamine are determined. In 300 mcg of sodium hyaluronate all compounds (except amino acids) are at non-detectable levels compared to the standard applied to the plate, as follows:

Glucosamine:	0.5 mcg
Glucuronic acid:	5.0 mcg
N-Acetylglucocamine:	5.0 mcg
Amino acids:	0.5 mcg

The sodium hyaluronate produced in accordance with the present invention preferably has a molecular weight within the range of about 600,000-700,000, as measured on an automatic Haney Viscometer, and a protein content of less than about 0.4% (Lowry), and exhibits a U.V. extinction coefficient of E_1^1 (257) nm) = less than 0.230; and E_1^1 (290

nm) = less than 0.190. The pH is preferably within the range of 7.5-7.7.

The assay of hyaluronic acid is based on the measurement of stoichiometric quantities of glucuronic acid released from hyaluronic acid following acid hydrolysis which should be not less than 97% and not more than 102%, calculated on the dry weight.

An alternant assay method may be used based on the electrophoretic identity test (A) after elusion of the alcian blue stained hyaluronic acid band with glacial acetic acid and reading of the O.D. at 670 nm against a standard, of not less than 97% and not more than 102%, calculated on the dry weight.

A preferred analytical method involves electrophoretic identification and assay of hyaluronic acid material which uses:

ELVI or suitable similar electrophoresis apparatus; cellulose acetate strips 10 x 2.7 cm;

alcian blue stain (Color Index 74240) 1% solution in 0.01 N hydrochloric acid, recently prepared;

a washing solution: HCl 0.01 N solution;

a buffer solution: pH 8.6, ionic strength 0.025; diethylbarbituric acid and its sodium salt.

A 10 ul of an aqueous solution of test substance containing 2 mg/ml, band-fashion, is deposited at the negative pole of the strip. Electrophoresis is carried out at 300 V for 20 minutes. The strip is removed and dipped in the alcian blue solution for 5 minutes. The unfixed dye is washed away by repeated washings with 0.01 N HCl solution. The result is a single band (comparable with a suitable standard) of blue color, representing hyaluronic acid, as illustrated in Figure 1.

Mucopolysaccharides other than hyaluronic acid can be detected by the previously described electrophoretic procedure. For instance, in addition to the characteristic blue band of sodium hyaluronate, the strip may show a weak

band with a higher Rf, as exemplified in Figure 2. In this regard, Figure 2 displays two bands instead of one: the first is the sodium hyaluronate band resulting from the 10 ul seeding; the second, less evident, connotes a mucopolysaccharide other than sodium hyaluronate, namely chrondroitin sulfate, from a 0.4 ug seeding. The sodium hyaluronate produced in accordance with the present invention should preferably have no more than 0.5% mucopolysaccharide other than hyaluronic acid.

Inasmuch as substantially all mucopolysaccharide that may be present as impurities are sulfurated mucopolysaccharides containing approximately 10% SO_4^{--}, assaying for SO_4^{--} constitutes an indirect method for the detection of these impurities.

In this regard, the organic matter is oxidized to form Na_2SO_4 followed by SO_4^{--} by precipitation with $BaCl_2$ and reading the turbidity.

In a pyrex test tube about 20 mg of test material is weighed and added to 1 ml of sulfate-free, 70% $HClO_4$; the test tube is permitted to stand in a sand bath at 180°C with a glass refluxing bubble, shown in Fig. 3, until mineralization is complete. Subsequently, the test tube is dried over a flame, cooled after which the residue is redissolved with a few drops of concentrated HCl and dried again to the complete disappearance of hydrochloric and nitrous fumes. The test tube with its contents is again cooled and the residue redissolved with 2 ml of 1N HCl; after which 2 ml of 5% BaI_2 solution is added. The resultant solution is agitated and permitted to stand for 30 minutes at room temperature; the extinction at 660 nm is read against a reagent blank. Inasmuch as 100 ug of SO_4^{--} gives an extinction reading of 0.180, this test must give an extinction not exceeding 0.018, representing 0.5 mg of sulfurated mucopolysaccharide in 100 mg (0.5%) of test product (upper limit).

The following methodology is used to determine the

presence of iron, lead, free glucuronic acid, n-acetylglucosamine:

To detect the presence of iron, 100 mg of Na-HA was accurately weighed and dissolved in 10 ml of 1N HCl. The absorption at the appropriate wave length is read using a suitable atomic absorption instrument against similarly prepared standards. The hyaluronic fraction (Na-HA) in accordance with the present should contain more than 100 ppm of iron.

To detect the presence of lead, 100 mg of Na-HA was accurately and dissolved in 10 ml of 1N HCl. The absorption at the appropriate wavelength is read using a suitable atomic absorption instrument against similarly prepared standards. The Na-HA in accordance with the present invention should contain no more than 10 ppm of lead.

The following procedure is used to detect the presence of free glucuronic acid, N-acetylglucosamine and glucosamine.

On a Kieselgel 60 plate (Merck No. 5721), 0.5 mcg of glucosamine, 5 mcg of N-acetylglucosamine and 300 mcg of sodium hyaluronate are deposited, and subsequently developed with ethanol-water solvent (67.33) containing ninhydrin 50 mg/100 ml and heat the plate to 100°C. Glucosamine will develop as a pink spot with an Rf of approximately 0.02, if other pink spots of various Rf appear, they represent various amino acids. The spots representing glucuronic acid (Rf ca. 0.65) and N-acetylglucosamine (Rf ca. 0.7) are visualized using iodine vapor (sensitivity 5 mcg).

The Na-Ha should test so that no evidence of the presence of any of the components is present. Therefore, free glucuronic acid, N-acetylglucosamine and glucosamine are present in Na-Ha of the present invention at concentrations less than the sensitivity of the method.

For purposes of the present invention, the molecular

weight of Na-HA is determined to be within the range of 600,000 to 700,000 using a Haney Automatic Viscometer. A typical procedure for doing so involves forming a 0.05M solution of sodium sulfate and deionized water, filtering 2 liters of the 0.05M sodium sulfate solution using a vacuum filter flask before transferring 1 liter of the filtered 0.05M sodium sulfate solution into a solvent controller. 2ml of Na-HA is then added to 100 ml of the 0.05 sodium sulfate solution to result in a standard solution. The standard solution is then injected into a Differential Viscometer (Model 100) and the Differential Viscometer is calibrated at 30° C according to this standard solution. A sample to be analyzed is then injected into the Differential Viscometer and the viscosity is computed based on the data using an IBM PC.

Using the method of Lowry, the protein content of Na-Ha of the present invention should be less than 0.4% using bovine serum albumin as the standard.

The assay for protein using the Lowry method involves first preparing working standards by diluting Protein Standard Solution with water to a volume of 1.0ml in appropriately labeled test tubes:

Protein Standard Solution (ml)	Water (ml)	Protein Concentration (ug/ml)
0.125	0.875	50
0.250	0.750	100
0.500	0.500	200
0.750	0.250	300
1.000	0	400

A test tube is labeled "Blank" and 1.0 ml water is added to the "Blank" test tube. Other samples are then added to appropriately labeled test tubes and diluted each to 1.0 ml with water. 1.0 ml Lowry Reagent Solution is then added to each tube and mixed well. The solutions are permitted to stand at room temperature for 20 minutes,

followed by rapid and immediate mixing while adding 0.5 ml Folin & Ciocalteu's Phenol Reagent Working Solution to each tube.

Color is allowed to develop for 30 minutes, after which the solutions are transferred to cuvets and the absorbance of the standards and samples vs. the blank are measured at a wavelength between 500 and 800nm with the readings being completed within 30 minutes.

The absorbance values of the standards vs. their protein concentrations are plotted to prepare a calibration curve.

The protein concentrations of the other tubes are determined from the calibration curve. The result is multiplied by the appropriate dilution factor to obtain the protein concentration in the original sample.

Characterization of polymers and Na-HA in accordance with the present invention may be accomplished using solution viscosity, preferably using a Hanley Automatic Viscometer. This is the fastest and easiest means of measuring polymer molecular weight on a relative basis. The differential measurement is based on a fluid analog of the Wheatstone Bridge. The differential pressure across this bridge is monitored continuously and at completion of run, a computer calculates the intrinsic viscosity. Molecular weight, in turn, is calculated using intrinsic viscosity and polymer constants.

The following are descriptions of assay methods used in accordance with the present invention.

The colormetric assay of mucopolysaccharides, consists of the colormetric determination of the glucuronic acid moiety of the mucopolysaccharide molecule. For purposes of the present invention the assay for sodium hyaluronate, a mucopolysaccharide, consists of a colormetric determination of the glucuronic acid portion of the sodium hyaluronate molecule. The assay is run using a suitable glucuronic

acid.

The assay is run in duplicate using a suitable glucuronic acid standard (STD), to accommodate small variations of extinction due to differences in heating and cooling times. To prepare the standard glucuronic acid solution, 52 mg of D-glucuronic acid salt, 99% (Aldrich), which contains 889.3 mg D-glucuronic acid per gram, is weighed out into a 100 ml volumetric flask and diluted with water followed by mixing and sonicating for 3 minutes. A 10.0 aliquot is pipetted into a 100 ml volumetric flask, diluted to volume with water, mixed and sonicated for 3 minutes to result in a concentration of 46.2 mcg. D-glucuronic acid per ml.

For the sodium hyaluronate test material, an aqueous solution containing sodium hyaluronate 100 mcg/ml is prepared by consecutive dilutions. This involves weighing out 10 mg of sodium hyaluronate standard into a 100 ml volumetric flask, diluting with water and sonicating for 3 minutes to result in a 100 mcg. sodium hyaluronate/ml solution. A 1.0 ml aliquot of sodium hyaluronate is pipetted into a volumetric flask diluted to volume with water, mixed and sonicated for 3 minutes.

For the standard glucuronic acid solution (BDH 38039), an aqueous solution containing glucuronic acid 46.2 mcg/ml. must be freshly prepared, again by consecutive dilutions.

Reagent A is prepared by dissolving 1.9 g of sodium tetraborate in 100 ml of concentrated sulfuric acid. This reagent is stable for several months in the refrigerator.

Reagent B is prepared by dissolving 125 mg of carbazole in 100 ml of absolute ethanol. This reagent is stable for two weeks when stored in an amber glass bottle in the refrigerator.

In a series of 6 test tubes, 10 ml of reagent A is added; all the test tubes are placed in an ice-water bath. To the first flask, 2.0 ml water is added to form a "Blank" test tube, a 2.0 ml portion of sodium hyaluronate standard

solution is added. To another two tubes, 2.0 ml portions of glucuronic acid standard solution, are added. To another two tubes, 2.0 ml portions of sample preparation, are added. The test tubes are stoppered before first agitating gently and then agitating vigorously. The test tubes are then transferred to a boiling water or steam bath using the refluxing apparatus shown in Fig. 3, or suitable alternative, for 10 minutes, then cool down in ice water. 0.5 ml of reagent B is added and agitated vigorously before heating for 15 minutes in the bath until a red coloration develops. The test tubes are then cooled in water to room temperature before reading the extinction versus the blank at 530 nm using a Beckman Spectrophotometer Model DU-7HS.

The color is stable for at least two hours.

The extinction readings of test and standard materials should be approximately the same, since the amount of glucuronic acid in the test material is similar to the standard, as indicated by the following formula:

$$\text{Na hyaluronate \%} = \frac{\text{Extinction test material}}{\text{Extinction standard}} \times 100$$

Alternately this may be expressed as

$$\frac{Asp}{Astd} \quad \frac{\text{wt Glucuronic Acid Std}}{46.397} \qquad 0.8893(10)=\text{mg/ml Na-HA}$$

where Asp = absorbance of samples and

Astd = absorbance of glucuronic acid standard

0.8893 = factor to correct glucuronic acid solution, 99% in terms of pure glucuronic acid

As used herein, pure sodium hyaluronate contains 46.297% glucuronic acid.

The sodium hyaluronate test substance is assayed using an electrophoretic technique by depositing 10 ul of an aqueous solution of sodium hyaluronate (2 mg/ml) on three electrophoretic strips, in parallels with another three strips seeded with standard sodium hyaluronate. After staining and washing, as previously described, excess fluid is removed from the strips with filter paper and let dry

completely at room temperature. The area containing the band is cut from each strip and placed in a test tube containing 2 ml of glacial acetic acid. The test tubes are placed in a water bath at 30-40°C and agitated occasionally to insure fine dispersion. 1 ml of water is added before agitating again. The solution obtained should be clear and light-blue colored. The color is stable and obeys the Lambert-Beer law for sodium hyaluronate concentrations between 5 and 40 mcg; the residence time of the strip in the stain solution is not critical as prolonged extraction results in negligible color change.

The OD of the solution is determined a suitable spectrophotometer at 620 nm against a blank obtained by identical processing of a strip area far from the band. The blank should read between 0.020 and 0.040. The extinction values of the bands, representing the 20 mcg standards, should be between 0.240 and 0.260, using the following formula:

$$\% \text{ Na-HA} = \frac{\text{Extinction (test material)}}{\text{Extinction (standard)}} \times 100$$

(where the extinction value is the mean OD of 3 solutions)

The precision and accuracy of electrophoretic assay may be calculated as follows:

The precision is calculated as the % coefficient of variation as follows:

$$\text{C.V.\%} = \frac{S}{X} \times 100$$

Where S = Standard Deviation, and
X = mean value of readings taken (12 readings in groups of 3). The standard deviation is the square root of the variance, or mean square. The variance (S^2) is obtained by dividing the sum of the squares of the scatters from the

mean by the number of degrees of freedom (n-1), where n is the number of determinations used for the calculation.

$$\text{Precision} = \frac{1.1615}{98.97} \times 100 = 1.173\%$$

The accuracy is calculated as:

$$\% \text{ deviation from} = \frac{\text{Theor. value} - \text{mean found}}{\text{Theor. value}} \times 100 = 1.03$$

The following is a characterization of the sodium hyaluronate (Na-Ha) in accordance with the present invention:

Na-HA is a clear, colorless, viscous aqueous solution.

The presence of Na-HA is determined using the electrophoretic method. The characteristic band of HA at a comparable Rf to standard should be visualized with alcian blue.

Glucuronic acid, glucosamine and N-acetylglucosamine are identified by TLC of an acid hydrolyzed samples of Na-HA with visualization using ninhydrin and iodine vapor.

The presence of sodium is detected in the sulfated ash of HA using atomic absorption spectrophotometry.

The pH of HA is within the range including 7.5 - 7.7 (electrometric).

The absence of impurities such as sulfated mucopolysaccharides and amino acids and degradation products such as glucuronic acid, glucosamine and N-acetylglucosamine in an unhydrolyzed sample are confirmed using the TLC method, wherein such impurities were not detected.

The assay of HA is based on the measurement of stoichiometric quantities of glucuronic acid released from HA following acid hydrolysis using the carbazole reaction,

i.e., between 97 and 102% of the labeled amount.

The fill volume meets the requirements of the U.S.P. XX for excess fill volume (page 8670).

The absence of pyrogens meets the requirements of the U.S.P. XX (page 907), using the contents of 1 vial per rabbit.

The sterility of Na-HA meets the requirements of the U.S.P. XX (page 878).

The content of methyl paraben is determined by U.S.P. XX TLC method or a suitable alternative procedure.

The following is a preferred procedure for preparing cosmetic formulations from purified sodium hyaluronate obtained by the previously described method in accordance with the present invention.

In a clean stainless steel container equipped with a stirrer and thermostat, 250 l of double distilled water and the methyl paraben is introduced and heated to about 60°C-90°C. Sodium hyaluronate is then added until it is completely dissolved, but preferably for at least 4 hours, after which the mixture is cooled to about 25°C while slowly stirring. The pH should be adjusted with 1N NaOH or 1N HCI, as required, to between 7.5 - 7.7 after which water for injection (U.S.P.) is added to a total volume of about 300 1. The solution is then filtered under nitrogen pressure through a Pall AXP 0.8 micron membrane and then fill into 30 ml Type 1 (Braun) colorless neutral glass bottle.

A preferred cosmetic formulation is:

Sodium Hyaluronate	3000 g
Methyl Paraben	600 g
Water for Injection (U.S.P.)	300 l

Laboratory tests have shown that the formula for treatment of skin conditions in accordance with the present invention is more pure than the other forms of Hyaluronate-based skin care products.

The HPLC-Gel-Exclusion Method is used for the

determination of sodium hyaluronate concentration in the formulation of the present invention compared with other formulations containing sodium hyaluronate which are commercially available. The procedure involves weighing out 1.0 gm sodium hyaluronate accurately into a 100 ml volumetric flask which is mixed until completely dissolved to result in a concentration of 10 mg per ml. 2.0 ml of HA stock solution is pipetted to 10 ml volumetric flask and q.s. to volume with water and mixed thoroughly.

2.0 ml solutions of the formulations containing sodium hyaluronate are pippetted to 10 ml volumetric flask and q.s. to volume with water and mixed thoroughly.

A liquid chromatograph is started up as per parameters and fun at a flow rate of 1.5 ml/min until a stable baseline is established. So as to ensure against ambient temperature fluctuation the column is insulated using suitable material and the room temperature is maintained as steady as possible.

A pilot run is required to determine retention time of external standard by injecting 20 mcl of pilot run. 20 mcl of external standard is then injected and an electronic integrator is calibrated.

Calculations can also be done by area under the curve as follows:

$$\frac{\text{Area Under Peak Sample}}{\text{Area Under Peak Standard}} \times 100 = \% \text{ HA Concentration}$$

The results of the comparisons of the formulation containing sodium hyaluronate in accordance with the present invention with commercially available formulations are shown in Figs. 4 and 5.

The present invention is, therefore, directed to the provision and use of a purified, substantially pyrogen-free, heat sterilized fraction of hyaluronic acid (HA) and the production of formulations and compositions containing such fraction of HA useful for cosmetic treatment of human tissue to alleviate the signs of aging or trauma caused by

burns or abrasions. The present invention is based on the discovery of a fraction of hyaluronic acid (HA) which is both safe and effective in the treatment of various skin conditions, for example the striae caused by aging or excessive environmental exposure, as well as the treatment of tissue which has been abraded or burned.

In cases where wind or too much sun has caused damage to the skin, including dryness, the formulations for treatment of skin conditions in accordance with the present invention should be applied several times daily to restore the skin to its normal condition within a few days. To rejuvenate and restore skin, showing signs of aging, such as, dryness, lines, rough patches and pigment changes, the formulations for treatment of skin conditions in accordance with the present invention should be applied to freshly cleansed skin one or twice daily. The formulations for treatment of skin conditions in accordance with the present invention may be used as a foundation for make-up or as an overnight treatment. Regular use of the formulations for treatment of skin conditions in accordance with the present invention is, therefore, believed to help maintain a youthful appearance and protect skin against harmful environmental factors which can accelerate the skin aging process.

For preferred results, the formulations for treatment of skin conditions in accordance with the present invention should be applied liberally for the first 2 or 3 weeks until the skin has absorbed the hyaluronate nourishment it requires. Thereafter, a small amount should be applied as discussed below. Other agents of fine quality, such as collagen, elastin or liposomes may be applied directly onto skin after the formulation for treatment of ski conditions in accordance with the present invention has been applied.

More preferably, the formulations for treatment of skin conditions in accordance with the present invention should be applied with a cotton tipped swab to freshly

cleansed skin of the face and neck before applying night cream or moisturizer, twice daily, at bedtime and in the morning.

It is believed that the advantages and improved results furnished by the methods and products of the present invention are apparent from the foregoing description of the preferred embodiment of the invention. Various changes and modifications may be made without departing from the spirit and scope of the invention as described in the claims that follow.

CLAIMS

1. A formulation for treatment of skin conditions comprising:

a) a fraction of hyaluronic acid obtained from a natural source;

b) preservatives; and

c) water.

2. The formulation as defined by claim 1, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9.

3. The formulation as defined by claim 2, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated mucopolysaccharides on a total weight basis;

iii) less than about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7.

4. The formulation as defined by claim 3, wherein said fraction of hyaluronic acid fraction has an average molecular weight within the range of 625,000-675.000.

5. The formulation as defined by claim 3, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

6. The formulation as defined by claim 3, wherein said fraction of hyaluronic acid is present in the form of a salt.

7. The formulation as defined by claim 6, wherein said salt is a sodium salt.

8. The formulation as defined by claim 7, wherein said sodium salt is sodium hyaluronate.

9. The formulation as defined by claim 8, wherein said preservative is selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of sodium benzoate, methylparaben, and propylparaben.

10. The formulation as defined by claim 9 wherein said preservative is methyl paraben.

11. The formulation as defined in claim 10, wherein said formulation comprises on a total weight basis:

1-1.2% sodium hyaluronate;

0.2-0.25% methyl paraben; and

98-99% water.

12. The formulation as defined in claim 11, wherein said formulation comprises on a total weight basis:

about 1.0% sodium hyaluronate;

about 0.2% methyl paraben; with the remainder being water.

13. A fraction of hyaluronic acid comprising at least one characteristic selected from the group of the following:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9.

14. A fraction of hyaluronic acid as defined by claim 13 wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated mucopolysaccharides on a total weight basis;

iii) about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7.

15. The fraction of hyaluronic acid as defined by claim 14, wherein said fraction of hyaluronic acid fraction has an average molecular weight within the range of 625,000-675,000.

16. The fraction of hyaluronic acid as defined by claim 15, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

17. The fraction of hyaluronic acid as defined by claim 16, wherein said fraction hyaluronic acid is present in the form of a salt.

18. The fraction of hyaluronic acid as defined by claim 17, wherein said slat is a sodium salt.

19. The fraction of hyaluronic acid as defined by claim 18, wherein said sodium salt is sodium hyaluronate.

20. A method of cosmetically treating skin comprising topically applying a formulation comprising:

a) a fraction of hyaluronic acid,
b) a preservative; and
c) water;

wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150ppm iron on a total weight basis;

v) less than about 15ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9, said fraction of hyaluronic acid being present in said formulation in an amount when applied to be effective to safely treat skin conditions selected from the group consisting of strial caused by aging or environmental exposure, abraded tissue and burned tissue.

21. The method of treating skin in accordance with claim 20, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated

mucopolysaccharides on a total weight basis;

iii) less than about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7.

22. The method of treating skin in accordance with claim 21, wherein said fraction of hyaluronic acid fraction has an an average molecular weight within the range of 625,000-675,000.

23. The method of treating skin in accordance with claim 21, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

24. The method of treating skin in accordance with claim 21, wherein said fraction hyaluronic acid is present in the form of a salt.

25. The method of treating skin in accordance with claim 24, wherein said salt is a sodium salt.

26. The method of treating skin in accordance with claim 25, wherein said sodium salt is sodium hyaluronate.

27. The method of treating skin in accordance with claim 26, wherein said preservative is selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of sodium benzoate, methylparaben, and propylparaben.

28. The method of treating skin in accordance with claim 27, wherein said preservative is methyl paraben.

29. The method of treating skin in accordance with claim 28, wherein said formulation comprises on a total weight basis:

1-1.2% sodium hyaluronate;

0.2-0.25% methyl paraben; and

98-99% water.

30. The method of treating skin in accordance with claim 29, wherein said formulation comprises on a total weight basis:

about 1.0% sodium hyaluronate;

about 0.2% methyl paraben; with the remainder being water.

31. A process for providing a fraction of hyaluronic acid from a natural source of hyaluronic acid comprising:

a) providing tissue containing a fraction of hyaluronic acid;

b) subdividing said tissue into particles;

c) subjecting said tissue particles to acetone extraction comprising:

i) mixing said particles of tissue with acetone to produce an acetone-tissue particle mixture;

ii) permitting said acetone-tissue particle mixture to settle; and

iii) removing supernatant acetone from said tissue particles;

d) continuing said acetone extraction to form supernatural acetone containing less than about 5% moisture;

e) removing moisture from extracted tissue particles to produce a dried acetone-extracted powder;

f) introducing papain to form an enzyme-buffer-tissue mixture;

h) filtering said enzyme-buffer-tissue mixture to a liquid;

i) ultrafiltrating said liquid to obtain an ultrafiltrate having a volume which is at least five times greater than the volume of said enzyme-buffer-tissue mixture;

j) continuing said ultrafiltrating until permeate is negative for amino acids;

k) reducing said volume of said ultrafiltrate to produce a concentrated liquid;

l) forming a substantially glycoprotein-free precipitate containing cetylpyridinium chloride;

m) subjecting said substantially glycoprotein-free precipitate containing cetylpyridinium chloride to extraction with a sodium chloride solution to recover extract supernatant containing hyaluronic acid;

n) precipitating impurities from said extract supernatant to produce a substantially clear, aqueous solution;

o) ultrafiltering said substantially clear, aqueous solution at a substantially constant volume to form a resultant filtrate;

p) concentrating said filtrate by ultrafiltrating said filtrate using a membrane with exclusion limits of about 300 DDS to remove low molecular weight hyaluronic acid to produce a concentrate;

q) precipitating said concentrate in alcohol;

r) washing resultant precipitate with an anhydrous acetone to form a substantially solid precipitate material;

s) drying said substantially solid precipitate material containing said fraction of hyaluronic acid.

32. The process for providing a fraction of hyaluronic acid in accordance with claim 31, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9.

33. The process for providing a fraction of hyaluronic acid in accordance with claim 32, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated mucopolysaccharides on a total weight basis;

iii) less than about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a ph within the range of 7.5-7.7.

34. The process for providing a fraction of hyaluronic acid in accordance with claim 33, wherein said fraction of hyaluronic acid fraction has an average molecular weight within the range of 625,000-675,000.

35. The process for providing a fraction of hyaluronic acid in accordance with claim 34, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

36. The process for providing a fraction of hyaluronic acid in accordance with claim 35, wherein said fraction hyaluronic acid is present in the form of a salt.

37. The process for providing a fraction of hyaluronic acid in accordance with claim 36, wherein said salt is a sodium salt.

38. The process for providing a fraction of hyaluronic acid in accordance with claim 37, wherein said sodium salt is sodium hyaluronate.

39. The process for providing a fraction of hyaluronic acid in accordance with claim 38, wherein said preservative is selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of sodium benzoate, methylparaben, and propylparaben.

40. A process for preparing a formulation containing a fraction of hyaluronic acid comprising:

a) forming an aqueous mixture at a temperature within the range of about $60^{o}C$-$90^{o}C$ comprising a fraction of

hyaluronic acid and water into a resultant solution;

b) adjusting the pH of said resultant solution to produce a pH adjusted solution comprising a fraction of hyaluronic acid, preservatives and water wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9; and

c) supplying said pH adjusted solution to containers.

41. The process for preparing a formulation as defined by claim 40, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated mucopolysaccharides on a total weight basis;

iii) about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7.

42. The process for preparing a formulation as defined by claim 41, wherein said fraction of hyaluronic acid fraction has an an average molecular weight within the range of 625,000-675,000.

43. The process for preparing a formulation as defined by claim 42, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

44. The process for preparing a formulation as defined by claim 43, wherein said fraction hyaluronic acid is present in the form of salt.

45. The process for preparing a formulation as defined by claim 44, wherein said salt is a sodium salt.

46. The process for preparing a formulation as defined by claim 45, wherein said sodium salt is sodium hyaluronate.

47. The process for preparing a formulation as defined by claim 46, wherein said preservative is selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of sodium benzoate, methylparaben, and propylparaben.

48. The process for preparing a formulation as defined by claim 47, comprising diluting said pH adjusted

solution with an amount of water to a final volume aqueous formulation comprising:

1-1.2% sodium hyaluronate;

0.2-0.25% methyl paraben; and

98-99% water.

49. The process for preparing a formulation as defined by claim 48, wherein said final volume aqueous formulation comprises on a total weight basis:

about 1.0% sodium hyaluronate;

about 0.2% methyl paraben; with the remainder being water.

50. The process for preparing a formulation as defined by claim 40, wherein said forming comprises:

i) providing a fraction of hyaluronic acid;

ii) introducing a preservative selected from the group consisting of methyl parahydroxybenzoate, propyl parahydroxybenzoate, sodium benzoate and mixtures of methyl parahydroxybenzoate, propyl parahydroxybenzoate and sodium benzoate into water heated to a temperature within the range of about 60°C-90°C to form a preservative solution;

iii) adding and dissolving said fraction of hyaluronic acid in said preservative solution to form said resultant solution.

51. The process for preparing a formulation as defined by claim 50, wherein said preservative is methyl paraben.

52. The process for preparing a formulation as defined by claim 51, wherein said fraction of hyaluronic acid is provided by a process comprising:

a) providing tissue containing a fraction of hyaluronic acid;

b) subdividing said tissue into particles;

c) subjecting said tissue particles to acetone extraction comprising:

i) mixing said particles of tissue with acetone to produce an acetone-tissue particle mixture;

ii) permitting said acetone-tissue particle mixture to settle; and

iii) removing supernatant acetone from said tissue particles;

d) continuing said acetone extraction to form supernatural acetone containing less than about 5% moisture;

e) removing moisture from extracted tissue particles to produce a dried acetone-extracted powder;

f) introducing papain to form an enzyme-buffer-tissue mixture;

h) filtering said enzyme-buffer-tissue mixture to a liquid;

i) ultrafiltrating said liquid to obtain an ultrafiltrate having a volume which is at least five times greater than the volume of said enzyme-buffer-tissue mixture;

j) continuing said ultrafiltrating until permeate is negative for amino acids;

k) reducing said volume of said ultrafiltrate to produce a concentrated liquid;

l) forming a substantially glycoprotein-free precipitate containing cetylpyridinium chloride;

m) subjecting said substantially glycoprotein-free precipitate containing cetylpyridinium chloride to extraction with a sodium chloride solution to recover extract supernatant containing hyaluronic acid;

n) precipitating impurities from said extract supernatant to produce a substantially clear, aqueous solution;

o) ultrafiltering said substantially clear, aqueous solution at a substantially constant volume to form a resultant filtrate;

p) concentrating said filtrate by ultrafiltrating said filtrate using a membrane with exclusion limits of about

300 DDS to remove low molecular weight hyaluronic acid to produce a concentrate;

q) precipitating said concentrate in alcohol;

r) washing resultant precipitate with an anhydrous acetone to form a substantially solid precipitate material; and

s) drying said substantially solid precipitate material containing said fraction of hyaluronic acid.

Amendments to the claims
have been filed as follows

1. A formulation for treatment of skin conditions comprising:

a) a fraction of hyaluronic acid obtained from a natural source;

b) preservatives; and

c) water.

2. The formulation as defined by claim 1, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9.

3. The formulation as defined by claim 2, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated mucopolysaccharides on a total weight basis;

iii) less than about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7.

4. The formulation as defined by claim 3, wherein said fraction of hyaluronic acid fraction has an average molecular weight within the range of 625,000-675.000.

5. The formulation as defined by claim 3, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

6. The formulation as defined by claim 3, wherein said fraction of hyaluronic acid is present in the form of a salt.

7. The formulation as defined by claim 6, wherein said salt is a sodium salt.

8. The formulation as defined by claim 7, wherein said sodium salt is sodium hyaluronate.

9. The formulation as defined by claim 8, wherein said preservative is selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of sodium benzoate, methylparaben, and propylparaben.

10. The formulation as defined by claim 9 wherein said preservative is methyl paraben.

11. The formulation as defined in claim 10, wherein said formulation comprises on a total weight basis:

1-1.2% sodium hyaluronate;

0.2-0.25% methyl paraben; and

98-99% water.

12. The formulation as defined in claim 11, wherein said formulation comprises on a total weight basis:

about 1.0% sodium hyaluronate;

about 0.2% methyl paraben; with the remainder being water.

13. A fraction of hyaluronic acid comprising at least one characteristic selected from the group of the following:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9.

14. A fraction of hyaluronic acid as defined by claim 13 wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated mucopolysaccharides on a total weight basis;

iii) about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7.

15. The fraction of hyaluronic acid as defined by claim 14, wherein said fraction of hyaluronic acid fraction has an average molecular weight within the range of 625,000-675,000.

16. The fraction of hyaluronic acid as defined by claim 15, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

17. The fraction of hyaluronic acid as defined by claim 16, wherein said fraction hyaluronic acid is present in the form of a salt.

18. The fraction of hyaluronic acid as defined by claim 17, wherein said slat is a sodium salt.

19. The fraction of hyaluronic acid as defined by claim 18, wherein said sodium salt is sodium hyaluronate.

20. A method of cosmetically treating skin comprising topically applying a formulation comprising:

a) a fraction of hyaluronic acid,
b) a preservative; and
c) water;

wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150ppm iron on a total weight basis;

v) less than about 15ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9, said fraction of hyaluronic acid being present in said formulation in an amount when applied to be effective to safely treat skin conditions selected from the group consisting of strial caused by aging or environmental exposure, abraded tissue and burned tissue.

21. The method of treating skin in accordance with claim 20, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated

mucopolysaccharides on a total weight basis;

iii) less than about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7.

22. The method of treating skin in accordance with claim 21, wherein said fraction of hyaluronic acid fraction has an an average molecular weight within the range of 625,000-675,000.

23. The method of treating skin in accordance with claim 21, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

24. The method of treating skin in accordance with claim 21, wherein said fraction hyaluronic acid is present in the form of a salt.

25. The method of treating skin in accordance with claim 24, wherein said salt is a sodium salt.

26. The method of treating skin in accordance with claim 25, wherein said sodium salt is sodium hyaluronate.

27. The method of treating skin in accordance with claim 26, wherein said preservative is selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of sodium benzoate, methylparaben, and propylparaben.

28. The method of treating skin in accordance with claim 27, wherein said preservative is methyl paraben.

29. The method of treating skin in accordance with claim 28, wherein said formulation comprises on a total weight basis:

1-1.2% sodium hyaluronate;

0.2-0.25% methyl paraben; and

98-99% water.

30. The method of treating skin in accordance with claim 29, wherein said formulation comprises on a total weight basis:

about 1.0% sodium hyaluronate;

about 0.2% methyl paraben; with the remainder being water.

31. A process for providing a fraction of hyaluronic acid from a natural source of hyaluronic acid comprising:

a) providing tissue containing a fraction of hyaluronic acid;

b) subdividing said tissue into particles;

c) subjecting said tissue particles to acetone extraction comprising:

i) mixing said particles of tissue with acetone to produce an acetone-tissue particle mixture;

ii) permitting said acetone-tissue particle mixture to settle; and

iii) removing supernatant acetone from said tissue particles;

d) continuing said acetone extraction to form supernatural acetone containing less than about 5% moisture;

e) removing moisture from extracted tissue particles to produce a dried acetone-extracted powder;

f) introducing papain to form an enzyme-buffer-tissue mixture;

h) filtering said enzyme-buffer-tissue mixture to a liquid;

i) ultrafiltrating said liquid to obtain an ultrafiltrate having a volume which is at least five times greater than the volume of said enzyme-buffer-tissue mixture;

j) continuing said ultrafiltrating until permeate is negative for amino acids;

k) reducing said volume of said ultrafiltrate to produce a concentrated liquid;

l) forming a substantially glycoprotein-free precipitate containing cetylpyridinium chloride;

m) subjecting said substantially glycoprotein-free precipitate containing cetylpyridinium chloride to extraction with a sodium chloride solution to recover extract supernatant containing hyaluronic acid;

n) precipitating impurities from said extract supernatant to produce a substantially clear, aqueous solution;

o) ultrafiltering said substantially clear, aqueous solution at a substantially constant volume to form a resultant filtrate;

p) concentrating said filtrate by ultrafiltrating said filtrate using a membrane with exclusion limits of about 300 DDS to remove low molecular weight hyaluronic acid to produce a concentrate;

q) precipitating said concentrate in alcohol;

r) washing resultant precipitate with an anhydrous acetone to form a substantially solid precipitate material;

s) drying said substantially solid precipitate material containing said fraction of hyaluronic acid.

32. The process for providing a fraction of hyaluronic acid in accordance with claim 31, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9.

33. The process for providing a fraction of hyaluronic acid in accordance with claim 32, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated mucopolysaccharides on a total weight basis;

iii) less than about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a ph within the range of 7.5-7.7.

34. The process for providing a fraction of hyaluronic acid in accordance with claim 33, wherein said fraction of hyaluronic acid fraction has an average molecular weight within the range of 625,000-675,000.

35. The process for providing a fraction of hyaluronic acid in accordance with claim 34, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

36. The process for providing a fraction of hyaluronic acid in accordance with claim 35, wherein said fraction hyaluronic acid is present in the form of a salt.

37. The process for providing a fraction of hyaluronic acid in accordance with claim 36, wherein said salt is a sodium salt.

38. The process for providing a fraction of hyaluronic acid in accordance with claim 37, wherein said sodium salt is sodium hyaluronate.

39. The process for providing a fraction of hyaluronic acid in accordance with claim 38, wherein said preservative is selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of sodium benzoate, methylparaben, and propylparaben.

40. A process for preparing a formulation containing a fraction of hyaluronic acid comprising:

a) forming an aqueous mixture at a temperature within the range of about 60°C-90°C comprising a fraction of

hyaluronic acid and water into a resultant solution;

b) adjusting the pH of said resultant solution to produce a pH adjusted solution comprising a fraction of hyaluronic acid, preservatives and water wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of:

i) a molecular weight within the range of 500,000-800,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-asetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9; and

c) supplying said pH adjusted solution to containers.

41. The process for preparing a formulation as defined by claim 40, wherein said fraction of hyaluronic acid exhibits at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 600,000-700,000;

ii) less than about 1% sulphated mucopolysaccharides on a total weight basis;

iii) about 0.4% protein on a total weight basis;

iv) less than about 100 ppm iron on a total weight basis;

v) less than about 10 ppm lead on a total weight basis;

vi) less than 0.00166% glucosamine;

vii) less than 0.0166% glucuronic acid;

viii) less than 0.0166% N-asetylglucosamine;

ix) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7.

42. The process for preparing a formulation as defined by claim 41, wherein said fraction of hyaluronic acid fraction has an an average molecular weight within the range of 625,000-675,000.

43. The process for preparing a formulation as defined by claim 42, wherein said fraction of hyaluronic acid contains less than 0.5% sulphated mucopolysaccharides on a total weight basis.

44. The process for preparing a formulation as defined by claim 43, wherein said fraction hyaluronic acid is present in the form of salt.

45. The process for preparing a formulation as defined by claim 44, wherein said salt is a sodium salt.

46. The process for preparing a formulation as defined by claim 45, wherein said sodium salt is sodium hyaluronate.

47. The process for preparing a formulation as defined by claim 46, wherein said preservative is selected from the group consisting of sodium benzoate, methylparaben, propylparaben, and combinations of sodium benzoate, methylparaben, and propylparaben.

48. The process for preparing a formulation as defined by claim 47, comprising diluting said pH adjusted

solution with an amount of water to a final volume aqueous formulation comprising:

1-1.2% sodium hyaluronate;

0.2-0.25% methyl paraben; and

98-99% water.

49. The process for preparing a formulation as defined by claim 48, wherein said final volume aqueous formulation comprises on a total weight basis:

about 1.0% sodium hyaluronate;

about 0.2% methyl paraben; with the remainder being water.

50. The process for preparing a formulation as defined by claim 40, wherein said forming comprises:

i) providing a fraction of hyaluronic acid;

ii) introducing a preservative selected from the group consisting of methyl parahydroxybenzoate, propyl parahydroxybenzoate, sodium benzoate and mixtures of methyl parahydroxybenzoate, propyl parahydroxybenzoate and sodium benzoate into water heated to a temperature within the range of about 60°C-90°C to form a preservative solution;

iii) adding and dissolving said fraction of hyaluronic acid in said preservative solution to form said resultant solution.

51. The process for preparing a formulation as defined by claim 50, wherein said preservative is methyl paraben.

52. The process for preparing a formulation as defined by claim 51, wherein said fraction of hyaluronic acid is provided by a process comprising:

a) providing tissue containing a fraction of hyaluronic acid;

b) subdividing said tissue into particles;

c) subjecting said tissue particles to acetone extraction comprising:

i) mixing said particles of tissue with acetone to produce an acetone-tissue particle mixture;

ii) permitting said acetone-tissue particle mixture to settle; and

iii) removing supernatant acetone from said tissue particles;

d) continuing said acetone extraction to form supernatural acetone containing less than about 5% moisture;

e) removing moisture from extracted tissue particles to produce a dried acetone-extracted powder;

f) introducing papain to form an enzyme-buffer-tissue mixture;

h) filtering said enzyme-buffer-tissue mixture to a liquid;

i) ultrafiltrating said liquid to obtain an ultrafiltrate having a volume which is at least five times greater than the volume of said enzyme-buffer-tissue mixture;

j) continuing said ultrafiltrating until permeate is negative for amino acids;

k) reducing said volume of said ultrafiltrate to produce a concentrated liquid;

l) forming a substantially glycoprotein-free precipitate containing cetylpyridinium chloride;

m) subjecting said substantially glycoprotein-free precipitate containing cetylpyridinium chloride to extraction with a sodium chloride solution to recover extract supernatant containing hyaluronic acid;

n) precipitating impurities from said extract supernatant to produce a substantially clear, aqueous solution;

o) ultrafiltering said substantially clear, aqueous solution at a substantially constant volume to form a resultant filtrate;

p) concentrating said filtrate by ultrafiltrating said filtrate using a membrane with exclusion limits of about

300 DDS to remove low molecular weight hyaluronic acid to produce a concentrate;

q) precipitating said concentrate in alcohol;

r) washing resultant precipitate with an anhydrous acetone to form a substantially solid precipitate material; and

s) drying said substantially solid precipitate material containing said fraction of hyaluronic acid.

Published 1990 at The Patent Office, State House, 66 71 High Holborn, London WC1R 4TP. Further copies may be obtained from The Patent Office. Sales Branch, St Mary Cray, Orpington, Kent BR5 3RD. Printed by Multiplex techniques ltd. St Mary Cray, Kent, Con. 1/87